SECTION I

Introduction and Program Summary

INTRODUCTION

The Genetics Research Center that would be enabled by this grant will be a new level of cooperative involvement of the Departments of Genetics and Pediatrics of the Stanford University School of Medicine in research in medical genetics and the application of such research to clinical aspects of medical genetics. We are requesting support for an interrelated set of basic and applied research projects involving members of both departments. Besides their importance as basic science, these investigations relate to genetic counselling, pathogenesis and diagnosis of genetic disorders and the mechanisms of human adaptation in genetic variation. In preparing this application, the two departments, labelled as basic science and clinical science respectively, have already enhanced the coordination of their activities for the benefit of patients with genetic disorders and their families.

Since 1959, the inception of the School of Medicine on the Stanford University campus, Genetics and Pediatrics each have been deeply involved in various aspects of genetic research. A number of ad hoc cooperative efforts have also evolved. Both departments are together teaching medical genetics to medical students. Informal communications in training graduate students and postdoctoral fellows have been excellent. Research projects undertaken in one department have involved consultation or collaboration with various members of the other department, and clinical problems have stimulated basic research activities in both departments.

In accordance with its clinical mission Pediatrics has focused on patients with genetic disorders, diagnosis, pathogenesis, and therapy; and the counselling of such patients and their families. Pediatrics has likewise emphasized perinatal biology, featuring a program of detection and management of high risk pregnancies in which the potential for morbidity or mortality of mother, fetus and/or newborn infant is high.

Genetics is well known for its basic research programs in molecular biology, population genetics, and immunogenetics, whose potential for clinical applications have long been recognized by members of both departments. As knowledge in molecular biology has increased and the necessary technology has permitted applications to the study of man and his disorders, it has become increasingly evident that relevant research efforts of both departments should converge on problems connected with clinical care. Both departments have begun to cooperatively design and carry out research applicable to genetic medicine and to plan for the application of the findings to the care of patients encountered on the various clinical services of the Department of Pediatrics.

The underlying theme that unifies the range of specific studies outlined below is genetic polymorphism in man. The pediatrician views this as the source of genetic disease; the basic scientist as an expression of gene mutation and evolutionary pressures. These are roles shared within as well as among individual investigators. To these challenges are brought a combination of clinical insights, experience with several aspects of basic genetics, and new analytical technologies -- the application of instruments like the mass-spectrometer, the computer and the cell sorter.

Besides the specific research projects to be funded under the Center grant, we work in an extensive context of genetic and related research -- and indispensable aspect of our own environment, and a set of activities to which the Center organization may also bring a new focus for developments that should advance both basic scientific knowledge and its application to human problems.

COMMENTS ON ORGANIZATION

The Stanford Genetics Research Center is organized as a cooperation of the Departments of Genetics and Pediatrics. Thus, it follows, rather than conflicts with existing departmental authority. This minimizes the need for new, formal arrangements that might be essential in other circumstances. It does reflect a deep-seated intention on the part of both departments to improve the application of new analytical methodology and of basic genetic science to clinical problems, and to relate clinical studies to enhancing knowledge of genetic polymorphism in man. The length of the following comments on organizational detail may lead one to believe that we are more interested in management than in scientific substance.

Exactly the opposite is the case, but we cannot afford the consequences of misunderstanding about how we will work together. Obviously, as at any other university, the actual character of our "organization" has a larger compass and bears little resemblance to the formal hierarchy of imputed power. Research is actually done in individual laboratories under the supervision of an autonomous faculty member. Most "direction" must be in the form of intellectual stimulation and criticism, and in the selection of key people for roles of autonomous responsibility.

Professor Joshua Lederberg, Chairman of the Department of Genetics, will serve as Principal Investigator and Director of the overall Genetics Center Program. Dr. Howard Cann, Associate Professor of Pediatrics, will serve as Associate Program Director with special responsibility for clinical research related to the Center's activities. (If this application is approved, and offers a funding basis for the step, it is anticipated that Dr. Cann will receive a joint appointment in the Genetics Department as well.)

The Director will have executive responsibility for the administration of the program, including the formulation of extended and new projects, their budgets, and reports to the NIGMS of progress under the grant, and internal functions of coordination, information and criticism.

The internal administration of the program is facilitated by the Director's position as Chairman of the Genetics Department, and by the enthusiastic support and participation of Dr. Irving Schulman, Chairman of Pediatrics, and of other senior members of both departments. In practice, the Director will be advised by, and will delegate subproject responsibility to, these colleagues in accordance with their special skills and interests.

The Director will also invite an informal visiting committee, principally from among his colleagues involved in similar lines of work at other West Coast institutions, to visit Stanford annually and to advise him of advantageous directions of

policy. This group may also help to identify new opportunities for inter-institutional cooperation and coordination, especially to further our emphasis on building complementary rather than competitive capabilities. These annual visits may also advantageously coincide with a local or regional conference on research progress concerning genetic polymorphism and disease.

The Director must play both an attractive and a critical role. That is he must encourage his colleagues to invest in the effort needed to orient their activities towards the common goals of the Center's research. At various times this attraction may be enhanced by the fiscal support of the Center's budget; on the other hand, it is hard to predict what the level of that support will be, and the level of discretion that will be delegated to the Director. Realistically speaking, then, his role is more inspirational than directive, at least with respect to the tenured fellow-members of the faculty. If the Center's budget is approved, and develops some de facto continuity, he may have greater formal authority -- but always with accountability to outside review groups and to the independent authority of other professors. The moral and intellectual influence that the Director can exert will obviously be greatest in relation to people in the same or closely co-functioning departments who already relate in many other ways. His main positive role will be to help bring in new ideas -- his own and others -- into the progress of the Center's research.

Conversely, he has responsibilities for negative functions -- especially to discourage the growth or even survival of projects that are inherently unsound, or become obsolete. Within a close-knit institution there are often serious personal and political obstacles to the exercise of objective scientific quality judgments. These limitations are both aggravated and compensated for by the limited discretion usually given to a director, i.e., the fact that final decisions about project items are generally made by peer review groups.

All of these problems would be much worse if the Director had the responsibility of coordinating every genetics-relevant activity in the school. He would have neither the budget nor the political mandate to do this well. In fact, the present proposal encompasses a serious and soul-searching effort to initiate a major Center program at a practically achievable and useful level of integration. It will also be the basis for less formal but equally important co-operations with other departments, whose policies and programs can be influenced but not directed in the exercise of our responsibilities.

Provision for Succession of Directorship.

Day to day deputization will be handled ad hoc in the same fashion as the duties of the departmental chairman. The question of possible succession, in the event of incapacity or transfer of the Director, poses a more difficult question. The Associate Director will serve pro-tem, in concert with the (acting)

chairman of each department; and a new director will be proposed by the Dean of the school after consultation with the visiting committee and with the participating faculty of the departments.

The Associate Director's role will be to maintain contacts with other clinical departments for patient referals to Center projects, and to assure a high standard of clinical responsibility in all Center research activities that deal with patients. Stanford has long experience with Clinical Research Centers -- e.g. in medicine, in cancer therapy, in cardiovascular disease, and in premature infant research. (These facilities afford further research bed opportunities for protocols involving intensive study of selected cases without burdening the present proposal with inappropriate in-patient care charges). In addition, they have led to the institutionalization of formal procedures for the ethical review of research involving human subjects. Dr. Cann will be responsible for defending the present proposal before that review committee, and for assuring that all further research under this grant is properly submitted and reviewed according to established procedures.

An Application for renewal of a Genetics Research Training Grant was approved by Council for the period 1974-1979. This action was superseded, however, by the executive decision to wind down such programs and we are now operating on a decreasing budget to cover only the previously enrolled trainees.

That training grant renewal had, for the first time, provided for clinical research in genetics as part of our program. Lacking these funds, it is, of course, imperative that the research activities of the Center also double as training opportunities for graduate research assistants and postdoctoral fellows (research associates.) We will make every effort to fit each fellow into employment in the support roles of the Center. We cannot pretend, however, to be able to support the training functions at the level previously assumed without a compensatory expansion of the funding for these research programs. This is not reflected in the budgets submitted at this time.

The department faculties having agreed to the programs of the Center, there is much to do in substance, but little in organizational process, to coordinate our activities and insure meeting the Center's objectives. We are already in frequent, almost daily, communication in the pursuit of our other duties and socially. These contacts are promoted by the fact that our laboratories and offices are immediately adjacent in the Medical Center Building.

The Genetics Center Budget is intended to fund programs of new substance and scope or those for which other support (e.g. NASA) is no longer available. It goes into new territory beyond many on-going research projects which are identified in the material on each affiliated investigator. However, the coordinated funding and administration of the Center allows more careful planning and resource-sharing than would be possible for

a disparate set of individual proposals.

We intend to minimize the indefinite prolongation of autonomous projects merely to keep a research group intact. Instead we will promote an innovative search for new opportunities and on-going mutual scrutiny of projects.

For the continuity of a program as complex as the Center, we are requesting an award for 5 years. Yet it is obvious that many unforeseen obstacles and opportunities will arise in that interval to warrant changes of tactical direction and evolution of major strategies. The responsibility of the Director to manage this progression of emphasis is his most important executive role. It will be discussed with the visiting committee and reported to NIGMS from year to year, to assure the consistency of his decisions with the approved mandate of the Center grant.

This is not an assertion of unwarranted latitude. To the contrary, the political structure of a university ensures, if anything, a great deal of conservatism in changes of direction and support for individual programs, once approved.

The present proposal will also be a model for further developments to be initiated with the Departments of Gyn-Ob., Medicine, Psychiatry and Dermatology -- to mention only those with which we have had tangible discussions. For various reasons, potential projects with them are not yet ripe for formal submissions; these will be the subject of future requests, depending in part on the climate for funding expansion of genetic research. Meanwhile, we have working arrangements to assure their awareness of our activities and the provision of patient material for mutual advantage.

A brief description of the role of each participant in this program and of his research and/or clinical interests are presented here:

THE STANFORD ENVIRONMENT

Besides the Program Director (Professor Lederberg) and Associate Director (Professor Cann) the faculty and professional personnel associated with the Center, their roles and biographical detail are spelled out in Section IX.

In addition, we have included biographical sketches of a number of key members of the environment of the Department of Pediatrics and Genetics, although their research projects are not included in funding under the Center at this time.

Within one or two years after the activation of this program, we anticipate the direct participation of one or more colleagues from the Department of Obstetrics and Gynecology. A search for a chairman of this department is presently under way, and we expect that this individual will renew active research

here in fetal physiology and fetal monitoring. We look forward to interacting in this program with our obstetrical colleagues in various projects pertaining to antenatal detection of genetic disorders and selective abortion.

We have also consulted with the chairmen of the Departments of Medicine and of Dermatology, and have encountered great interest in the development of the Center, and assurances of cooperation in the referal of patients who would be pertinent to our screening technologies. Their position is best witnessed by the attached correspondence. At some future date, we may expect to formulate more specific proposals, as a consequence of recruiting now under way.

The Department of Psychiatry will be chaired by Albert Stunkard (now at University of Pennsylvania) beginning September 1973. He is personally well known to the Director (who served on the search committee for this appointment, which was chaired by Professor Schulman) and many other participants and we have strong assurances that the Psychiatry Department's interest in genetic etiologies of psychiatric disease will continue under his leadership. Dr. Stunkard has also voiced his concern that psychiatrists have not hitherto been more directly involved in problems of genetic counseling, like those reflected in Dr. Barnett's proposal here; and we are looking forward to closer cooperation on such issues after his assumption of his duties. For some time, Professor Cavalli-Sforza of the Genetics Department has been cooperating with Professor Barchas of Psychiatry on polymorphisms of biogenic amine metabolism.

Genetic counseling, per se, is a responsibility mainly of the Pediatrics Department -- diseases of adults only occasionally present serious problems of reproductive policy of the family. Genetic disease, of course, presents itself to all of the medical and surgical specialities. In recent years, these departments have not seen any requirement for a special organization to deal with genetic aspects of internal medicine or surgery -- and indeed these are fully and competently integrated into their overall teaching and practice. When special problems do arise, there are no impediments to consultation with pediatricians and with basic geneticists. Many members of those departments are already involved in immunogenetic and other genetic research.

DATE: May 25, 1973

TO : Joshua Lederberg, Ph.D.

FROM : Daniel D. Federman, M.D. (Chairman, Department of Medicine)

SUBJECT: Genetics Research Center

Dear Josh:

I am not really clear about your timetable for submission of the proposal for a Genetics Research Center, but I thought I would make explicit several areas of potential involvement of the Department of Medicine.

1. Pharmacogenetics - We have a slot in Clinical Pharmacology for a new Assistant Professor. Stan Cohen and I have discussed finding someone with a particular interest in the area of pharmacogenetics, but at the present time, no particular individual has been chosen.

2. Endocrinology -

A. Hormonal control of gene expression. We will have at least one new person in the Endocrine Division in the next year and have placed as our first interest finding someone working in the area of the effective hormone on the expression of genetic information. This would provide an obvious link to a genetic center.

3. Early detection of hereditary endocrine disease. There are at least 80 endocrinopathies which show Mendelian segregation and a number more which probably have a major genetic control. Early detection of assymptomatic patients can be achieved by suitable provocative testing. This opens the way to family studies in which a) the hereditary mechanism can be defined, b) preventive or therapeutic intervention can be tried before irreversible damage has occurred, and c) the effects that intervention can be compared to the natural history of the disorder. The latter approach has important application to the management of "patients" discovered by multiphasic screening techniques.

4. Immunogenetics - This area of course is strongly represented in our Department and we could, I believe, contribute significantly to the activities of the genetic center in this area.

I should have liked to provide more specific information, but until particular people have been identified, it would be difficult to do so. I hope this is of some help -- I am certainly anxious for an active collaboration.

Sincerely,

DDF

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CLINIC FOR CHILDREN WITH PSORIASIS AND OTHER INHERITABLE SKIN DISEASES

Directors:

Eugene M. Farber, M.D.
Professor and Chairman, Department of Dermatology

Alvin H. Jacobs, M.D.
Professor of Dermatology and Pediatrics

In the clinics of the Department of Dermatology there are about 12,000 total patient visits annually, of these approximately 3,000 are children seen in the Pediatric Dermatology Clinic. Over half of these children have either psoriasis or atopic dermatitis, both conditions with important heritable factors in their etiology. In addition many patients are seen with the less common genodermatoses, such as, the various types of ichthyosis, epidermolysis bullosa, and the many types of heritable neurocutaneous disorders. At the present time a special clinic is being established for the care and study of children with psoriasis and other inheritable skin diseases. The purpose of this clinic is not only to offer more complete care for these types of patients, but to study the genetic aspects of these conditions and offer genetic councelling to the patients and their families.

The Department of Dermatology already has an unmatched reservoir of material for genetic study in its Psoriasis Life History Research files; a data-bank of approximately 8000 patient histories. Not only is this computerized information a source of information on the epidemiology of psoriasis, but has served as the basis for several published follow-up studies on the genetics of psoriasis including family and twin studies.

At present several genetic studies are under way. For example, Dr. Farber and associates are studying the association between H-LA antigens and psoriasis. Drs. Jacobs and Chan are studying and developing techniques for accurate counting of melanocytes in pigmented and depigmented macules in order to predict the development of certain genodermatoses, such as neurofibromatosis, tuberous

sclerosis etc.

Studies are also under way to properly classify the various genetic types of ichthyosis and in cooperation with Dr. Howard Cann we are planning to investigate the x-linked blood type in the families with x-linked ichthyosis.

It is hoped that in the future, with the vast resources available in Genetics, Pediatrics, and Dermatology at Stanford, an investigation could be launched into the genetic aspects of atopic dermatitis, one of the commonest skin problems of childhood.

CLINICAL FACILITIES

The Stanford Medical Center, located midway between the cities of San Francisco and San Jose on the campus of Stanford University, consists of the School of Medicine, the University Hospital and the Stanford Clinics. Stanford is the only university hospital in the Regional Medical Program (RMP) Area III and functions as a tertiary care center. RMP Area III consists of eleven counties in mid California consisting of 2.6 million people. There are 60 acute hospital facilities, and 4000 physicians practice in RMP Area III. Approximately 90 percent of patients admitted to Stanford University Hospital live in this area. Additionally, patients for the clinics and university hospital are also drawn from other areas in and out of California. RMP Area III and its population form a base from which is drawn numerous patients with diseases resulting from major genes or chromosomal abnormalities or with a significant genetic component. Most of these patients are seen primarily on one of the services of the Department of Pediatrics or are referred for diagnostic evaluation and/or genetic counselling from other clinical services at the medical center. Clinical teaching services at other hospitals* affiliated with the Department of Pediatrics add to the source of patients with medical genetic problems.

As the Genetics Center activities evolve and research developments become available for clinical application, we anticipate that more patients will be referred to this medical center for genetic counselling and evaluation and management of genetic disease. The number of patients seen annually by the combined clinical facilities of the Department of Pediatrics for genetic disorders is estimated to be about 200. We predict that this number will double during the course of the Genetics Center grant.

Other clinical divisions of the Medical Center, of course, also see many adult patients with genetic disorders. Historically, Genetics has had weaker ties with them than with Pediatrics, although several joint projects connect with Medicine (H. McDevitt) and with Psychiatry (J. Barchas; S. Kessler). The appointment (effective February 1) of a "genetic endocrinologist", Dr. Daniel Federman, as new head of Medicine should remove administrative obstacles to extensions of Center-related programs.

Patients with genetic disorders are seen in the following pediatric subspecialty clinics at the Stanford Medical Center.

*The Children's Hospital at Stanford and the pediatric services at the Santa Clara County Hospital (San Jose) and at the Kaiser-Permanente Medical Center in Santa Clara.

Genetic Counselling Clinic (Director, Dr. Howard Cann, Associate Professor of Pediatrics)

Approximately fifty patients and their families are referred annually for genetic counselling. These include patients from outside of the Stanford Medical Center and from various clinical services at the Medical Center. About 50% of families seen are counselled for disorders determined by major genes at single loci. The remaining 50% of patients represent more complex counselling problems, e.g. possible sporadic cases and phenocopies, multifactorial threshold traits, undiagnosed familial disorders, and excessive exposure to radiation. Counselling includes confirmation of the diagnosis of the disorder and identification of heterozygotes among asymptomatic relatives at risk (autosomal recessive and X-linked recessive disorders). Families are always screened for the possibility of applying ante-natal diagnostic techniques. The motivation for and expectations from genetic counselling are explored by Dr. Cann and, for selected families, by a social worker, in order to plan for effective communication with each family.

Birth Defects Clinics (Director, Dr. Luigi Luzzatti, Professor of Pediatrics)

Approximately 100 new patients are seen in the Birth Defects Clinics annually for diagnostic evaluation and comprehensive and interdisciplinary management of congenital defects. There are about 400 return visits per annum in the clinics. In addition, about 50 newborn infants are seen annually for evaluation of congenital defects in the Stanford Hospital Nurseries. Most children seen have multiple congenital defects of unclear etiology, about 50% of these are screened for chromosomal abnormalities, about 25% are patients with disorders determined by major genes at single loci, and about one third are seen for structural malformationswhich are multifactorially inherited threshold traits. This is the clinic where most of the patients with mucopolysaccharidoses, mucolipidoses, simply inherited disorders of bone, Down's syndrome and other chromosomal abnormalities, cleft lip and/or palate and congenital defects or neural tube development are seen. Where appropriate, genetic counselling is provided to families of patients enrolled in the Birth Defects Clinics. In addition about 50 families are referred each year specifically for genetic counselling. An average of about 100 karyotype analyses are done annually in the Cytogenetics Laboratory in probands with congenital defects and/or in family members as indicated. To date diagnostic amniocentesis has been performed at 12-16 weeks gestation on approximately 25 women referred to the clinic for either advanced maternal age or a documented family history for an X linked gene.

Pediatric Hematology Clinic (Clinic Director, Dr. Herbert C. Schwartz, Professor of Pediatrics)

In this clinic approximately 100 new patients are seen annually for various disorders of blood. Between five and ten percent of these are children with inherited disorders -- sickle cell anemia and other hemoglobinopathies, thalassemia, congenital spherocytosis, glucose-6-phosphate dehydrogenase deficiency and other instances of congenital non-spherocytic hemolytic anemia and hemolytic disease of the newborn (feto-maternal blood group incompatibility). In this clinic workup of patients with genetic disorders includes identification of heterozygotes and homozygotes among first degree relatives of probands and subsequent genetic counselling.

The Stanford Nurseries (Director, Dr. Philip Sunshine, Associate Professor of Pediatrics)

The newborn nurseries of the Stanford Medical Center include facilities for uncomplicated births, an intensive care nursery and the Premature Infant Research Center. The research and clinical emphasis of these newborn units is on perinatal biology of the fetus, newlyborn infant and the mother. This emphasis has led to the development of a program dealing with high risk pregnancies, in which mother, infant or both are at high risk for morbidity and mortality. Whenever appropriate the fetus is monitored and such monitoring includes diagnostic amniocentesis for evaluation of fetal maturity, for fetomaternal Rh incompatibility and early antenatal detection of genetic disorders. A study conducted in California in 1971 by the Bureau of Maternal and Child Health indicated that the Stanford Medical Center is the only facility in RMP Area III with an intensive care nursery (based on fairly ridgid criteria, e.g. full time neonatologists and basic infant monitoring equipment available at all times), and this is evident by referrals of fetuses and their mothers and newborn infants. Approximately 700 infants are admitted per year to either the intensive care nursery of the Premature Research Center at Stanford and about 30% of these are born at other facilities. Approximately 5% of newborn infants receiving intensive care at Stanford are ill because they are heterozygous or homozygous for a major mutant gene, because of a chromosomal abnormality or because of a malformation with a significant genetic contribution. We anticipate a major expansion of perinatal activities involving detection and management of high risk mothers and fetuses with the appointment in the near future of a Chairman of the Department of Obstetrics and Gynecology. The search is presently underway, and one of the criteria for selection is competence in the areas of fetal monitoring and evaluation and fetal and maternal physiologic interactions.

Pediatric Neurology Clinic (Clinic Director, Dr. Judith Koehler, Assistant Professor of Pediatrics and Neurology)

Approximately 500-600 patients with various neurologic disorders are seen annually in this clinic. Many of these patients have genetically determined or inherited disease, e.g. learning disorders, familial myopathies, spinocerebellar degeneration, familial spastic paraparesis and other metabolic or degenerative disorders of the central and peripheral nervous system. The Division of Pediatric Neurology has special interest in clinical treatments of and research into the basic mechanisms of epilepsy. Some patients with seizure disorders have heredofamilial determinants underlying their basic seizure problems. Additionally, response to medication and drug metabolism may be genetically determined. The Division of Pediatric Neurology also has special clinical and research interest in neuromuscular disease, much of which is hereditofamilial. Specifically for this purpose, a neuromuscular clinic is being established in conjunction with the Muscular Dystrophy Association. This clinic will see 200-300 patients, mostly children, annually. A diagnostic neuromuscular histochemistry laboratory is already in function to process nerve and muscle biopsies from these patients. Active research in neuromuscular disease is being carried out by several workers in the Department of Neurology which incorporates ultrastructure and basic physiologic studies.

Hemophilia Program (Clinic Director, Dr. John Gribble)

The Hemophilia Program provides diagnostic evaluation and comprehensive management for patients with disorders of blood coagulation. Approximately 100 new patients are seen annually; these patients represent the clinical spectrum of disorders of blood coagulation, the most frequent being hemophilia A, hemophilia B and von Willebrand's disease. The families of all patients with inherited disorders are provided with genetic counselling. A comprehensive care clinic is located at the Children's Hospital at Stanford in order to provide continuing care for patients with hemophilia and includes prophylactic treatment of patients with hemophilia A with Factor VIII preparations.

Pediatric Metabolic and Endocrine Clinic (Clinic Director, Dr. R.O. Christiansen, Assistant Professor of Pediatrics)

Seventy to eighty new patients are seen annually in this clinic which logs about 700 patient visits each year. Many of the patients may be classified as inborn metabolic errors and these include phenylketonuria, galactosemia, the various syndromes associated with adrenal hyperplasia, alcaptonuria, the various glycogenoses and defects in thyroxine biosynthesis. Diet therapy of patients with phenylketonuria and galactosemia is an ongoing activity of these clinics. Furthermore, a number of patients with hypophosphatemia (X-linked) are enrolled in this clinic. Genetic counselling is provided for the families of all patients with hereditary metabolic and endocrine diseases, and this includes attempts to designate heterozygotes among unaffected siblings of probands with inborn errors of metabolism.

Growth and Development Clinic (Director, Dr. Norman Kretchmer, Professor of Pediatrics)

This clinic deals with genetic and environmental problems in infants and children which affect their growth and development. Usually children are referred to this clinic because of failure to grow or abnormally slow growth. Thus children with hereditary conditions which result in significant growth failure in the phenotype (e.g. isolated growth hormone deficiency, various aminoacidurias and various chondrodystrophies) comprise a proportion of this clinic's patient population. Approximately 150 new patients are seen annually.

The Children's Hospital at Stanford, located approximately 1/2 mile from the Medical Center on the Stanford campus, is affiliated with the Department of Pediatrics for teaching and postdoctoral clinical and research training. There are three clinical services relevant to a medical genetics program at this hospital: 1) Cystic Fibrosis Service (Director, Dr. Birt Harvey, Clinical Associate Professor of Pediatrics), 2) Clinical Immunology Service (Director, Dr. Vincent Marinkovich, Assistant Professor of Pediatrics) and 3) Pediatric Oncology Service (Director, Dr. Jordan Wilbur, Clinical Associate Professor of Pediatrics). These services have in-patient and out-patient facilities at the Children's Hospital at Stanford. Here patients are evaluated, diagnosed and treated for cystic fibrosis, hereditary immune defects and hereditary malignant tumors (e.g. retinoblastoma). Genetic counselling is provided by the staff of these services.

Research Facilities

Research facilities in the Departments of Genetics and Pediatrics available to this Genetics Center Program include some 15,000 square feet of laboratories, research support areas and offices. The research and administrative areas for each department are contiguous on the third floor (the Joseph P. Kennedy Jr. Laboratories for Molecular Medicine) of the Joseph D. Grant Building of the School of Medicine. In addition the Instrumentation Research Laboratory (Department of Genetics), in the basement of this building, comprises about 7,000 square feet of offices, laboratories, and shops. Thus biochemical laboratories, fully developed tissue culture areas, heavy equipment areas, cold and warm rooms and an electron microscope facility are available for this program. A cell separator, a gas chromatograph, a mass spectrometer and an electron microscope lead the long list of equipment which is available for the research projects to be carried out in this program. Other equipment also includes centrifuges, scintillation counters, lyophilizers, spectrophotometers, incubators, microscopes and coulter counters. Rather good computer facilities are available.

THE RESEARCH PROGRAM

The Center program consists of research projects which have direct relevance to medical genetics. Some of these projects will require blood, urine, amniotic fluid and cells from patients and others will require the patients themselves. In none of these research projects are we ready to test methods for use in the actual clinical situation; rather, we are developing methodology. The general areas of research are as follows:

I. Screening and Characterization of Inborn Errors of Metabolism by Gas Chromatography/Mass Spectrometry Analysis of Body Fluids.
(Drs. Lederberg, Kretchmer, Cann and Duffield).

II. Maternal Blood Stream - Another Source of Fetal Tissue for Pre-Natal Diagnosis of Genetic Disorders.
(Drs. Herzenberg and Cann).

III. Polymorphic Genetic Markers in Amniotic Fluid.
(Drs. Cann and Tsuboi).

IV. A Search for Genetic Polymorphisms and Variances of Specific Binding Proteins in Blood.
(Dr. Cavalli-Sforza).

V. The Impact of Genetic Counseling Practices on Family Decisions and Behavior.
(Drs. Barnett, Cann and Luzzatti).

The arrangement is also reflected in the budget presentation.

SECTION II

Screening and Characterization of Inborn Errors of Metabolism by Gas Chromatography/Mass Spectrometry Analysis of Body Fluids

Drs. Lederberg, Kretchmer, Cann, and Duffield

Screening and Characterization of Inborn Errors of Metabolism with Computerized Gas Chromatography And Mass Spectrometry

Dr. J. Lederberg, Principal Investigator
Drs. N. Kretchmer, H. Cann, and A. Duffield,
Associate Investigators

A. INTRODUCTION

A.1 Objectives

The objectives of this work are to develop the uses of gas-liquid chromatography (GC) and mass spectrometry (MS) instrumentation, under computer management, for the screening, diagnosis (pre and postnatal), and study of inborn errors of metabolism. The efficacy of these analytical tools has been demonstrated when applied to limited populations of urine samples in the research laboratory environment. We propose to enlarge the clinical investigative applications of GC/MS technology and to demonstrate its utility for more economical and routine diagnosis and screening of disease.

Specific goals include the application of GC/MS analysis capabilities to larger and more diversified populations to establish better defined norms, deviations, and control parameters necessary to relate GC/MS analysis results to identifiable disease states. In order to ease the problems in analyzing the prodigious amounts of information expected in this research, we will augment the existing GC/MS data handling system to provide for increased throughput and automation.

Two other on-going or pending research projects relate to the present application, each with distinctive aims:

1. DENDRAL (NIH: RR-00612; Principal Investigator, E. A. Feigenbaum) is concerned with the advancement of artificial intelligence (computer software) techniques for the automated interpretation of mass spectrometry data. These programs attempt to emulate human reasoning processes in constructing explanations for mass spectra from basic principles.

2. SUMEX (NIH: RR-00785 pending; Principal Investigator, J. Lederberg) is a comprehensive resource grant to establish a national facility for developing applications of artificial intelligence in medicine. Our own use of this facility will include the integration of DENDRAL software with HIGH RESOLUTION mass spectrometry instrumentation. Genetic screening can be made even more sophisticated by using these techniques for the corroboration of the structures of newly discerned metabolites. However, the present program can also operate stand-alone, if necessary, using low resolution MS on line with the GC.

A.2 Background and Rationale

The Instrumentation Research Laboratory was established in the Genetics Department under NASA auspices in 1961. Its task was to define and improve microanalytical methods for the detection of living processes that might be useful for the biological exploration of the planets. Many of the concepts that we explored have been embodied in NASA's planetary mission plans. However, we have not undertaken to design and build hardware for such missions. Instead, we have served as experienced advisors to the experiment teams responsible for scientific studies on the Mariner and Viking Mars programs. Our work on GC/MS is one of several lines of instrumentation effort.

Our original mandate from NASA included generous encouragement to seek health-related applications as a spin-off of the development work they were supporting. However, they have not been able to support the full fledged extension of space-related technology to genetic disease research per se. The present application also comes at a time when overall funding for basic research by NASA is declining rapidly and may disappear within the next year. We have already begun to reduce our GC/MS laboratory staff in response to these cutbacks. It is therefore appropriate that we seek NIH support to help maintain this existing laboratory to apply its capabilities to problems of characterizing genetic metabolic disease.

Our focus on mass spectrometry (ref 3c) originally stemmed from the exquisite sensitivity, speed, and specificity of this technique for the identification of organic molecules. We have had some experience with instruments (like the Bendix Time-of-Flight Mass Spectrometer) which can generate a complete low resolution mass spectrum in 100 microseconds and whose sensitivity is limited by the statistics of the number of ionized fragments, and by the data handling problems of averaging repetitive spectra emerging at a rate of 10,000 frames per second. We have also been led to look at the computational challenges of MS from another standpoint - namely the mechanization of the scientific thinking that is entailed by the interpretation of a mass spectrum. This task has been the focus of the research in "artificial intelligence" of the DENDRAL project.

The application of these instruments to genetic research requires another dimension, namely the separation of complex mixtures, e.g., from body fluids, into individual components. These are then available for identification by the mass spectrometer. Gas chromatography has proven to be a useful companion to the mass spectrometer - the output gas stream can be fed directly to the inlet of the spectrometer and much of the carrier gas (helium) selectively deviated by a semi-permeable membrane. (Automated, continuous flow into a mass spectrometer of other chromatographic streams, e.g., high pressure liquid chromatography, is a speculation that may eventually materialize

to great advantage but is not yet available for applications like ours).

For some time then we have been developing the means to integrate GC with MS under computer management. The present project represents the systematic application of these skills to the recognition and identification of metabolic variations, viewed both as genetic polymorphisms and as clinical problems of genetic disease in man.

The sample populations comprise mainly healthy (control) and problematical newborns already under intensive study in the Stanford Pediatrics Department. Other samples will be furnished by collaborative arrangements with physicians elsewhere and with other Departments at Stanford (e.g., Medicine, Psychiatry, Dermatology, etc.). These inputs will have been prescreened for conditions likely to relate to possible genetic etiologies or otherwise to exercise the analytical utility of GC/MS screening.

The Techniques of MS and GC

The technique of mass spectrometry gives information about the structure of a molecular species by measuring the characteristic mass abundance pattern of fragments resulting from ionizing the parent molecule. Ionization is usually accomplished by electron bombardment. The compound under analysis must have a measurable vapor pressure at about 200 degrees C. (This temperature and a pressure of 0.01 microbars are the normal operating conditions of a GC-coupled mass spectrometer ion source). The ionizing electron beam (70 eV energy) removes one electron from some of the molecules of the sample vapor to yield excited positive molecular ions:

$$M + e \longrightarrow M(+) + 2e$$

The molecular ion, M(+), is generally unstable (especially if at a high energy of excitation) and may decompose within a few microseconds to yield a series of positively charged fragment ions. Each fragment ion can in turn decompose to ions of lesser

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Although the technique of mass spectrometry was extensively used by petroleum chemists from the 1940's, it was not widely utilized in organic chemistry until the late 1950's. The first extensive monograph on biochemical applications of mass spectrometry has just appeared ("Biochemical Applications of Mass Spectrometry," edited by G. R. Waller, Wiley-Interscience, New York, 1972). Our colleagues and close collaborators in the Stanford Chemistry Department, led by Professor Carl Djerassi, have been among the pioneers in the development of MS for natural product chemistry, especially as applied to steroids (4 books and in excess of 200 papers on various aspects of the theory and application of MS have been published by Prof. Djerassi's group since 1961). During the 1960's, mass spectrometry was applied to many different types of organic compounds. The accumulation of these reference mass spectra was necessary to establish fragmentation rules for the interpretation of unknown mass spectra. The experienced mass spectroscopist becomes adept at recognizing the mass spectral signatures of those types of compounds with which he works but he cannot encompass within his memory all the relevant information contained in the literature. In addition, many reference spectra determined by mass spectrometry laboratories have not been published. To overcome this problem, libraries of mass spectra are being compiled for computer storage and retrieval so that they will eventually be available for matching by computer against the mass spectra of unknown compounds. Progress is now being made toward compiling libraries of mass spectra relevant to general metabolic studies. These will match the accumulation already available for special classes of organic molecules and for some drugs whose spectra are important for emergency toxicological analyses (ref 2).

Instrumentation advances in mass spectrometry during the past decade like improved sensitivity, direct coupling with GC and the use of computers for the routine recording and presentation of mass spectra, all facilitate the large scale application of mass spectrometry to biomedical problems.

Body fluids and other materials encountered in biomedical research are complex mixtures. For example, urine is known to contain several hundred organic compounds at levels exceeding on the order of 1 nanogram per milliliter. The gas chromatograph is indispensable for the separation of such mixtures into discrete components. With medium resolution instruments, the mass spectrometer can be scanned repeatedly once every 2-4 seconds. The gas chromatographic separation of a urine mixture may require 40-50 minutes: the result is the accumulation of over 700 mass spectra per analysis. The simplest way to identify these mass spectra is to search a library of known compounds. Even if the mass spectrum of a test compound does not reside in the library, the best match found may be a related compound. This can facilitate the manual interpretation based on the chemist's knowledge of and guesses about the rules of fragmentation. The

problem of computerizing the identification of compounds whose mass spectra are not in a library is addressed by the DENDRAL project. Computer programs have been developed to interpret mass spectra of unknown compounds from first principles (i.e., to emulate the reasoning processes of organic chemists).

Frequently compounds of biochemical interest occur in small amounts in biological fluids. (By definition, many frontier problems concern compounds at the limit of easy detection by existing techniques). Thus, the effectiveness of GC/MS as a detector of biological materials is directly related to its sensitivity. Current systems routinely operate with sensitivities such that mixture components with as little as 50-300 nanograms of material can be measured. This limitation is imposed by the following instrument-related factors. In order to record an interpretable mass spectrum, a low resolution mass spectrometer must have input to its source on the order of 5 nanograms of material per second. Since a gas chromatographic peak lasts for approximately 5 to 30 seconds, in GC/MS operation some 25-150 nanograms per GC peak are required for mass spectral analysis. Inherent in the gas chromatographic column and in the semi-permeable membrane separator (used to preferentially remove the helium carrier gas from the effluent stream) are losses of up to 50% which increase the input sample requirements in a practical sense to about 50-300 nanograms of material per GC peak analysis.

Another limiting factor in the application of the GC/MS technique to biological extracts concerns the volatility of the material to be assayed. Before the system can detect many non-volatile components (e.g., carbohydrates, amino acids, etc.), they must be converted into volatile derivatives which will pass through the gas chromatograph at a maximum oven temperature of 300 degrees C. Above this temperature, column bleed from the gas chromatographic phase will tend to enter the ion source and complicate the recorded spectra. Thus the GC/MS technique is restricted to those organic compounds which can be converted to volatile derivatives and is not, in general, applicable to inorganic compounds. A recent report (ref 1) describes the analysis by GC/MS of ketose diphosphates (as their trimethylsilyl (TMS) derivatives) while aldose diphosphates (also as their TMS derivatives) proved to be too unstable to analyze. It is safe to assert, however, based on our own experience and the literature, that a broad spectrum of organic compounds of biological significance will be amenable to analysis by GC/MS methods.

There is another mode of operation of a GC/MS system which enables greater sensitivities to be attained for the quantitation of KNOWN metabolites. If the mass spectrum and the gas chromatographic retention time of the compound to be quantitated are known, the mass spectrometer can be used as a specific detecting system for this compound. This technique is called mass fragmentography. Under these experimental conditions, the mass spectrometer is not scanned over the ENTIRE mass range but is directed to measure one or two SPECIFIC masses known to be

characteristic of the compound(s) being quantitated. Consequently, there is an appreciable increase in sensitivity since the mass spectrometer samples only the significant data points and can integrate the signal longer.

In this mode, existing GC/MS instrumentation matches the new fluorescent reagents for amines (reported to detect approximately 10 picomoles). It also embodies the specificity of the mass spectrum at individual mass numbers. At greater cost and cumbersomeness, the MS can be extended to a quantum-counting range of sensitivity. These methods are therefore likely to be complementary to the special purpose methods, like fluorescimetry, which are often cheaper and more efficient for well defined classes of compounds. On the other hand, the history of pesticide analysis shows how the GC can also be made ultrasensitive at the cost of some loss of specificity.

Using deuterated analogs as standards for the test compound, quantitation can also be achieved at sub-nanogram levels. We have recently exploited certain characteristics of the quadrupole mass spectrometer and its data system to develop a method for the quantitation of ten amino acids in soil extracts (ref 3a, copy attached) and subsequently for the amino acid content of biological fluids (ref 3b). This represents an advance in the technique of mass fragmentography since the sector mass spectrometers used up to this time, have been severely limited in the number of ions and the mass range they could monitor for any one experiment. Our technique of quadrupole mass fragmentography was used for the quantitation of the amino acids in the urine of a patient with suspected branch chain amino aciduria. The results are discussed later (see Methods of Procedure and figure 5).

The overall management of the system and the reduction and selective presentation of the large volume of data emanating from the analytical instruments is an important task of the control computer. Our experience in instrumentation comprises a good deal of computational software embracing real time instrument management, automated data reduction, and artificial intelligence (ref 4, appended to this application). It also requires considerable effort in electronic and vacuum technology for the instrumentation hardware, and a coherent system approach for the overall integration of these components.

Present GC/MS systems are designed mainly for laboratory research, incorporating great analysis flexibility but the ability to handle only a small number of samples. Such systems are not practical for large volume screening, but they can be adapted for the pilot studies contemplated here. A properly designed automated system could reduce these costs by as much as an order of magnitude, as would be essential for cost-effective applications to general health care.

The routine screening of normal and abnormal body metabolites, including drugs, in human body fluids (ref 5) is currently the object of several research programs. Various

non-specific methods, including thin layer (ref 6,7), ion exchange (ref 8,10), liquid (ref 9), and gas chromatography (ref 11-14 and 17b), are used primarily with the goal of separating a large number of unnamed constituent materials. Using these techniques, compound "identification" is made by a comparison of the migration, under identical conditions, of the unknown spot with reference compounds. This approach can lead to erroneous identifications, however. This point is illustrated by a recent article (15) which describes the use of mass spectrometry in the identification of a case of isovaleric acidemia. Previously, the same patient was diagnosed as having butyric and hexanoic acidemia on the basis of chromatographic evidence alone. This type of error is especially important when analyzing a "new" (previously undescribed) metabolic error where rigorous identification of relevant metabolites in various body fluids and tissues is essential.

For positive identification using mass spectrometry, the separated components must be transferred from the chromatographic medium to the mass spectrometer. The unknown spot can be leached from paper or thin layer chromatograms, or in the case of liquid chromatography, the solvent removed, and a mass spectrum recorded on the residual material. Trapping the various effluent components from a gas chromatograph, with subsequent introduction into the mass spectrometer, has been used. This approach requires considerable time and is inefficient when applied to complex separations. It has been superseded by the direct coupling of the gas chromatograph and mass spectrometer.

Gas chromatography is unquestionably the most convenient separation technique to couple to the mass spectrometer because the carrier gas can be removed efficiently and easily as the analysis proceeds. For recent examples of the use of the GC/MS technique for the analysis of body fluids see refs 15-18. Based on the work cited, as well as our own on-going programs, the ability of the GC/MS technique for the analysis of body fluids is well established. We have drawn upon the published literature in helping to design our experimental protocols (ref 18).

As mentioned earlier, urine and other body fluids (e.g., serum, amniotic fluid, etc.) are complex mixtures. The separation (by gas chromatography) and subsequent identification (by mass spectrometry) of these components can be a difficult task. To simplify the separation problem, the body fluid under analysis is chemically separated into a number of fractions which permit analysis for acids, amino acids, and carbohydrates present in free or in conjugated form. Drugs and hormones, as well as their metabolites, will also appear in the chromatographic separations. The gas chromatographic analysis of each class of compounds presents a metabolic profile. Abnormal profiles (containing either novel peaks or peaks deviating from their expected amplitudes) are then assayed by mass spectrometry. The mass spectra recorded during the elution of each gas chromatographic peak then serve to identify the constituents present in that peak.

The importance of this diagnostic technology may be illustrated by a family seen for a fatal inherited disease at the Stanford Medical Center. Three male infants have been born to this couple and each of the offspring died in a very similar manner by 10 days of age. The first infant was not seen at Stanford, but the second infant was transferred from another hospital, in extremis. Aside from the striking family history, there were no diagnostic clues, and the second child was dead within 6 hours of arrival. During the period of observation, blood and urine were collected from the proband, and analysis of the latter by gas chromatography (at another institution) revealed an excess of orotic acid. This diagnostic clue, coupled with the clinical picture of relatively rapid demise after protein (breast milk) feedings were well established, resulted in a diagnosis of a hereditary deficiency of hepatic ornithine transcarbamylase, with resulting hyperammonemia (ref 19). This diagnosis was eventually confirmed when the third male infant of this couple behaved in a manner identical to that of his two deceased brothers and, despite attempts at therapeutic intervention which included low protein feedings, he developed documented hyperammonemia and died in this medical center. Study of the liver revealed the complete absence of ornithine transcarbamylase. It must be stressed that clinical observations of the first two infants and post mortem examinations were not helpful diagnostically, and it was the chemical identification of orotic acid which led to the understanding of the familial pattern of neonatal mortality in this family.

Most medical centers have access to amino acid analyzers in order to screen patients for metabolic abnormalities of the principal amino acids, but unless a special research interest exists, other inborn errors of metabolism cannot easily be studied. At this institution the GC/MS system provides us the opportunity to detect a wide variety of inborn errors which show accumulation of amino acids, fatty acids, and many other metabolites in urine, blood, amniotic fluid, and other biological fluids and tissues.

Another application of GC/MS pertains to pre-natal diagnosis of hereditary inborn errors of metabolism. Fetal urine contributes to the amniotic fluid by the twelfth week of gestation and it should provide information which is diagnostically relevant to the fetus (ref 20). Surprisingly little is known about the origin, fate, and components of normal amniotic fluid, although information is accumulating because of interest in pre-natal diagnosis and evaluation of the fetus (ref 21). The fetal cells of the amniotic fluid, cultivated IN VITRO, are used routinely for pre-natal diagnosis of genetic disorders (ref 22). The fluid itself has been used infrequently for diagnostic purposes, although there is increasing evidence for the utility of direct assay of this component of amniotic fluid for pre-natal diagnosis of heritable metabolic errors. Mahoney et al. (ref 23) have recently reported the first successful pre-natal diagnosis of methylmalonic aciduria (confirmed by study of the fetus and performed in time to elect abortion). Their

observations suggest that at 12 weeks of gestation, methylmalonic acid is undetectable in the amniotic fluid surrounding a normal fetus and, probably, a fetus who is heterozygous for the (recessive) gene (ref 24). Goodman, et al. (ref 25) have successfully diagnosed argininosuccinic aciduria in a 16 week fetus. This involved detection of argininosuccinic acid in the amniotic fluid plus enzymatic studies on cultured amniotic fluid cells. Normally this compound is not detected in amniotic fluid at 16 weeks of gestation. There are reports of other conditions being diagnosed in the fetus by direct assay of amniotic fluid (ref 26) as well.

The accuracy of diagnostic procedures probing amniotic fluid for soluble constituents has been questioned because of the possibility of contamination with maternal blood and because of lack of information on the normal state at various times of gestation. If the fluid component could be used for pre-natal diagnostic purposes, the phenotype of the fetus could be detected relatively rapidly as compared to the time required to culture sufficient amniotic cells. Hereditary diseases which are potentially amenable to diagnosis by analysis of the soluble constituents of amniotic fluid are those in which the accumulating metabolite is not cleared by the placenta but is expected to appear in fetal urine. Defects in epithelial transport, e.g., cystinuria and Hartnup disease, are examples of such conditions. However, it is clear that this class of metabolic errors is not the only one which might be detectable by direct assay of amniotic fluid. The examples provided above suggest that fetuses with overflow type metabolic errors may also be detected.

The development of diagnostic and screening techniques suitable for various inborn errors of metabolism will require a suitable computer based methodology for screening a large selected sample of subjects with the subsequent resolution of data into classifications describing normal states and ranges as well as specific correlations of GC/MS analysis abnormalities with disease states. With a modest augmentation of existing instrumentation facilities, we can accomplish these analytical tasks on increasing numbers of patients.

B. SPECIFIC AIMS

a) We plan to use GC/MS to screen urine and plasma from normal individuals of various ages, including premature and newborn infants, in order to establish adequate control data and to understand variations encountered.

b) We plan to use GC/MS in the diagnosis of inherited metabolic abnormalities and in the detection and study of previously unrecognized metabolic disorders.